

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Fan, Jian-Bing, et al.
Appl. No. : 09/779,376
Filed : February 07, 2001
Title : NUCLEIC ACID DETECTION
METHODS USING UNIVERSAL
PRIMING

Customer No.: 41552
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Grp./A.U. : 1634
Examiner: Lu, Frank Wei Min

DECLARATION UNDER 37 C.F.R. § 1.132

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Alexandria, VA 22313-1450

Sir:

I, Jian-Bing Fan, Ph.D., declare as follows:

1) I am a Director of Scientific Research at Illumina, Inc. (Illumina), where I have held this position for 7 years. Prior to my current position, I worked as a Senior Scientist from June 1999 to March 2000 and an Associate Director of Genetic Analysis from April 2000 to October 2000 at Illumina.

2) Prior to joining Illumina, I was the Manager of Polymorphism Research at Affymetrix, Inc. (Affymetrix). While at Affymetrix (1996 - 1999), I spearheaded the development of high-density oligonucleotide array technology and its application in large-scale Single nucleotide polymorphism (SNP) identification and genotyping. Before joining Affymetrix, I was a senior Research Fellow/postdoctoral fellow at Stanford Human Genome Center (1992 - 1996). I was a key scientist involved in all aspects of the Center's scientific operation, such as large-scale physical mapping and sequencing of human genome.

3) It has been explained to me that three requirements must be satisfied for a combination of prior art references to render obvious a claimed invention. First, the cited art must teach or suggest all the limitations of the invention as recited in the claims. Second, the cited art, couples with the knowledge generally available in the art at the time of the invention, must contain some suggestion or incentive to have motivated the ordinary skilled artisan to modify a reference or to combine references. Third, the proposed modification of the cited art must have had a reasonable expectation of success, to one ordinary skill in the art at the time the invention was made. I understand that the following factors are considered in making this determination: (1) the scope and content of the prior art; (2) the differences between the prior art and the claimed invention; (3) the level of skill in the pertinent art; and (4) secondary factors of unobviousness.

4) I obtained a Bachelor's of Science majoring in Biochemistry in 1982 from Fudan University, Shanghai, China, and a Ph.D. in Genetics in 1992 from Columbia University, New York. I have over 15 years of experience in nucleic acid detection, genome research, and microarray array technology development. I have served on the editorial board of *Genome Research*, a prestigious peer review journal in the genomics field since 2004. My research focuses on the genotyping of SNPs, gene expression profiling/splicing monitoring, and genome-wide DNA methylation detection. I have authored numerous papers in these areas. A copy of my curriculum vitae and a list of publications is attached as Exhibit 1.

5) I am a co-inventor on the application entitled "Nucleic Acid Detection Methods Using Universal Priming" having U.S. Serial No. 09/779,376, filed February 7, 2001. I have read the Office Action mailed April 5, 2007, and understand that claims 5, 13, 32, 39, 45 and 57 are rejected under 35 U.S.C. § 103(a) as obvious over Barany et al., U.S. Patent No. 6,534,293 ("Barany et al."), in view of Schneider et al., U.S. Patent No. 4,882,269 ("Schneider et al."). I have read and am familiar with both Barany et al. and Schneider et al.

6) I understand that the Examiner cites Barany et al. for describing nucleic acid detection methods using coupled ligase detection and polymerase chain reactions. Schneider et al. is cited for describing immobilizing a hybridization complex formed in solution to a solid support. The Examiner concludes that it would have been obvious to one of ordinary skill at the

time the invention was made to immobilize a complex described by Barany et al. to a solid support because immobilization would enhance separation of the complex from unhybridized probes and the signal generated from the immobilized complexes. The Examiner also concludes that one of ordinary skill also would have had a reasonable expectation of success.

7) For the reasons summarized in this paragraph and detailed in the paragraphs that follow, it is my opinion that, based on the cited references, the skilled person in the art would not have expected to combine a solid phase immobilization step of ligation complexes as described and claimed in the application to achieve the claimed results. The invention claims a method of determining a nucleotide at a detection position in a multiplex format where at least 96 different target sequences are assayed in a single reaction mixture. At the time the invention was made, multiplexing samples for nucleotide detection at this level was not viewed as possible. The ability to accurately and reproducibly detect at least 96 different nucleotide positions ("96plex") in a single reaction mixture was very much contrary to expectations. At the time, ordinary solution phase assays such as the one described by Barany et al. were used in a multiplex format, but only at very low levels of about 12-24 targeted nucleotide positions in a single assay. Therefore, achieving multiplex detection of at least 96 different nucleotide positions was very much considered a great advancement.

8) To demonstrate the extreme disparity between multiplex detection without the claimed immoboilization step and the multiplex method of the invention using attachment of ligation complexes to a solid phase I have performed a side-by-side comparison. Nucleotide detection assays were performed following the methods of the invention. A copy of the procedure employing immobilization of ligation complexes is attached as Exhibit 2. A copy of this procedure employed without the immobilization step is attached as Exhibit 3. The procedures are identical except for the first several steps. In Exhibit 2, genomic DNA was first annealed with oligonucleotide probes and immobilized onto a solid-support; several wash steps were performed to remove excess and mis-hybridized oligonucleotides; subsequent oligonucleotide probe extension and ligation reactions are carried out on solid-phase. While in Exhibit 3, the oligonucleotide probe annealing, extension and ligation reactions occur simultaneously in a reaction solution. These modifications to the protocol of Exhibit 3 were

mandated by, and incurred because of, the immobilization step in method of the invention (Exhibit 2).

9) The results of this side-by-side comparison are attached as Exhibits 4-9 and demonstrate that the claimed methods yield exceedingly high levels of call rate and reproducibility when at least 96 different detection positions are determined in a single reaction. In contrast, employing the same method, but without immobilization of the ligation complexes produced essentially random scattered data that cannot be used to make reliable genotype call.

9) Exhibit 4 is a summary of the results comparing the claimed method of the invention with or without immobilization of ligation complexes . Each of sixteen different genomic DNA (“gDNA”) samples was assayed for 1,536 different SNPs in a single reaction mixture. Therefore, for each gDNA sample, the multiplex level is 1,536. For each multiplex assay, Exhibit 4 provides the call rate, which is the fraction of correctly determined nucleotide identity at particluar positions (“genotype calls”) expressed as a fraction of all possible calls, and the concordance, which is a measurement for the degree of reproducibility (i.e., same genotypes reported by duplicated experiments). Experiments preformed in our assay without any genomic DNA input yield a call rate from randomly scattered data points of about 0.6 (data not shown). Therefore, call rates below 0.6 are considered to be akin to random background noise. The results shown in Exhibit 4 indicate that immobilization of the ligation complexes dramatically increases both the call rate and the reproducibility of each multiplex sample compared to the procedure without an immobilization step. The mean call rate and concordance was 0.548 and 0.783, respectively, without the immobilization, indicating that these multiplex determinations were essentially random. In contrast, immobilization of ligation complexes increased the mean call rate to 0.989 and the concordance to 0.982, respectively.

11) The above results are graphically illustrated in attached Exhibits 5-9 for five representative determinations within the 1,536 multiplex reaction. These graphs correspond to genotype clusters generated from the 16 individual DNA samples, assayed with an oligonucleotide probe pool corresponding to 1536 SNPs. Each of the Exhibits 5-9 shows a pair of plots for a single SNP measured for all of the 16 DNA samples in duplicate (hence, a total of 32 data points). The y-axis is normalized assay value (sum of intensities of the two channels,

Cy3 and Cy5, corresponding to the two respective alleles) and the x-axis is the "theta" value ($\frac{2}{\pi} \tan^{-1}(Cy5 / Cy3)$). Theta values near 0 are homozygotes for allele "A" and theta values near 1 are homozygotes for allele "B". The software automatically grouped the 32 data points for each SNP into the two homozygote clusters (AA and BB, respectively) and the heterozygote cluster (AB).

12) The left hand plots for each of Exhibits 5-9 shows data from multiplex SNP determinations employing the immobilization step, (i.e., the gDNAs were attached to a "solid-support"). The right hand plots for each of Exhibits 5-9 shows data for the same SNP measured in the same 16 gDNA samples except the gDNAs were not attached to a solid-phase, thus labeled "without immobilization."

13) The performance of clustering for the data points is a direct indication of the data quality. The "call rate" is measured as the percentage of data points within each of the three possible clusters. The "concordance" is measured as the percentage of duplicated measurements within the same cluster. The accuracy is measured by the distance that separates clusters.

14) As illustrated by the high level of clustering, all of the five representative multiplex determinations (Exhibits 5-9) show a pronounced degree of call rate, call accuracy and reproducibility when ligation complexes were immobilized (left graph) when compared to the determinations which did not immobilize the ligation complexes (right graph). Separation between homozygote and heterozygote clusters is excellent and variation within each cluster is small when the immobilization step was used (left graph). In contrast, the multiplex method which did not immobilize ligation complexes exhibits a wide spread of data points, illustrating the essentially random nature of the assay at this multiplexing level (right graph).

15) The above results demonstrate immobilization of ligation complexes for detection of at least 96 different nucleotide positions in a single reaction mixture exhibits unexpectedly superior performance compared to the same method performed without this step of the invention. These results were very surprising because, at the time the invention was made, multiplexing at this level was not contemplated as possible.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Date: 10/5/2007

By:



Jian-Bing Fan, Ph.D.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME Jian-Bing Fan	POSITION TITLE Director of Scientific Research		
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Fudan University, Shanghai, China	B.S.	1982	Biochemistry
Columbia University, New York	Ph.D.	1992	Genetics
Stanford University	Post-Doc	1996	Human Genetics

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Jian-Bing Fan, Director of Scientific Research, Illumina, Inc., has over 15 years of experience in human genome research and array technology development. His research focuses on the genotyping of single nucleotide polymorphisms (SNPs), gene expression profiling/splicing monitoring, and genome-wide DNA methylation detection. He is currently applying all these technologies in a broad spectrum of cancer studies with a special emphasis on biomarker discovery and validation. Prior to joining Illumina, Dr. Fan was the Manager of Polymorphism Research at Affymetrix. While at Affymetrix, Dr. Fan spearheaded the development of high-density oligonucleotide array technology and its application in large-scale SNP identification and genotyping. Before joining Affymetrix, Dr. Fan was a senior Research Fellow/postdoctoral fellow at Stanford Human Genome Center. He was a key scientist involved in all aspects of the Center's scientific operation, such as large-scale physical mapping and sequencing of human genome. Dr. Fan received his PhD in Genetics from Columbia University.

A. Positions and Honors.**PROFESSIONAL POSITIONS**

1996-1998	Staff Scientist	Genetic Research	Affymetrix, Inc.
1999	Manager	Genomics	Affymetrix, Inc.
6/99-3/00	Senior Scientist	Genomics	Illumina, Inc.
4/00-10/00	Associate Director	Genetic Analysis	Illumina, Inc.
10/00-present	Director	Scientific Research	Illumina, Inc.

AWARD

1986: Genetic Society of America Travel Award.
2002: Illumina Innovation Award.

Editorial Board: **Genome Research (2004 – 2007)**

EXHIBIT 1

PROFESSIONAL MEMBERSHIPS

American Association for the Advancement of Science (AAAS)

American Association for Cancer Research (AACR)

The Human Genome Organization (HUGO)

The American Society of Human Genetics (ASHG)

American Association for Clinical Chemistry (AACC)

B. Selected publications (in chronological order).

1. J.-B. Fan and D. Kuang. Functional expression of yeast *Saccharomyces cerevisiae* tRNA gene and structural gene (Pro 2) in *Escherichia coli*. Chinese Scientific Bulletin 18:1421-1422. 1986.
2. Smith, C.L., T. Matsumoto, O. Niwa, S. Klco, J.-B. Fan, M. Yanagida and C.R. Cantor. An electrophoretic karyotype for *Schizosaccharomyces pombe* by pulsed field gel electrophoresis. Nucleic Acids Research 15:4481-4489. 1987.
3. J.-B. Fan, Y. Chikashige, C.L. Smith, O. Niwa, M. Yanagida and C.R. Cantor. Construction of a *Not I* restriction map of the fission yeast *Schizosaccharomyces pombe* genome. Nucleic Acids Research 17:2801-2818. 1989.
4. J.-B. Fan, S.H. Korman, C.R. Cantor and C.L. Smith. *Giardia lamblia*: haploid genome size determined by pulsed field gel electrophoresis is less than 12 Mb. Nucleic Acids Research 19:1905-1908. 1991.
5. J.-B. Fan, D. Grothues and C.L. Smith. Alignment of *Sfi I* sites with the *Not I* restriction map of *Schizosaccharomyces pombe* genome. Nucleic Acids Research 19:6289-6294. 1991.
6. J.-B. Fan, M. Rochet, C. Gaillardin and C.L. Smith. Detection and characterization of a ring chromosome in the fission yeast *Schizosaccharomyces pombe*. Nucleic Acids Research 20:5943-5945. 1992.
7. T. Zhang, J.-B. Fan, S. Ringquist, C.L. Smith and C.R. Cantor. The 0.7 to 3.3 megabase chromosomes from *Candida*, *Kluyveromyces*, and *Pichia* provide accurate size standards for pulsed field gel electrophoresis. Electrophoresis 14(4):290-295. 1993.
8. C.L. Smith, S. Klco, T.Y. Zhang, H. Fang, R. Oliva, J.-B. Fan, M. Bremer and S. Lawrence. Analysis of megabase DNA using pulse-field gel electrophoresis. In "Methods in Molecular Genetics" (K.W. Adolph, Ed.), Academic Press, San Diego. 1993.
9. J.-B. Fan, J. DeYoung, R. Legace, R.A. Lina, Z. Xu, J.C. Murray, K.H. Buetow, J. Weissenbach, R.D. Goold, D.R. Cox and Richard M. Myers. Isolation of yeast artificial chromosome clones from 54 genetic loci located on human chromosome 4 at 1000:1 odds or greater. Human Molecular Genetics 3(2):243-246. 1994.
10. J.-B. Fan and C.L. Smith. Endogenous endonuclease hypersensitive sites in *Schizosaccharomyces pombe* chromosomes. Genetic Analysis 12(2):85-93. 1995.
11. N.E. Stone, J.-B. Fan, V. Willour, L. Pennacchio, J. Warrington, A. Hu, A.-E. Lehesjokil, A. de la Chapelle, D.R. Cox and R.M. Myers. Construction of a 750-kb bacterial clone contig and restriction map in the region of human chromosome 21 containing the progressive myoclonus epilepsy (EPM1) gene. Genome Research 6:218-225. 1996.
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13. E.A. Stewart, K.B. McKusick, A. Aggarwal, E. Bajorek, S. Brady, A. Chu, N. Fang, D. Hadley, M. Harris, S. Hussain, R. Lee, A. Maratukulum, K. O'Connor, S. Perkins, M. Piercy, F. Qin, T. Reif, C. Sanders, X. She, W.-L. Sun, P. Tabar, S. Voiticky, S. Cowles, J.-B. Fan, C. Mader, J. Quackenbush, R.M. Myers, and D.R. Cox. An STS-based radiation hybrid map of the human genome. Genome Research 7:422-433. 1997.
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- homology domain-containing insulin receptor binding protein hGrb10 γ . *Journal of Biological Chemistry* 272:29104-29112. 1997.
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Exhibit 2: Multiplex Detection with Immobilization of Ligation Complexes

Biotinylation of genomic DNA: Genomic DNA (5 µl at 50 ng/µl) was mixed with 5 µl biotinylation reagent (MS1; Illumina) and incubated at 95°C for 30 minutes. A precipitation reagent (PS1; Illumina; 5 µl) was then added, followed by addition of 15 µl of 2-propanol. The samples were mixed thoroughly and spun at high speed (3000 g). The pellets were resuspended in buffer (RS1; Illumina; 10 µl) and used in the subsequent assay step.

Annealing of oligonucleotide probes to genomic DNA: Genotyping oligonucleotides probes (corresponding to 1536 specific SNPs) and paramagnetic particles were combined with the activated DNA in the oligonucleotide/target annealing step, in which the oligonucleotides probes hybridize to the genomic DNA as it binds to paramagnetic particles. Two Allele-Specific Oligonucleotides (ASOs) and one Locus-Specific Oligonucleotide (LSO) were designed for each SNP (Fan et al., Highly parallel SNP genotyping, *Cold Spring Harb Symp Quant Biol*, 68:69-78 (2003)). All three oligonucleotide sequences contained regions of genomic complementarity and universal PCR primer sites; the LSO also contained a unique IllumiCode sequence complementary to a particular bead type. The IllumiCode sequence hybridizes to the universal bead type probes in the last hybridization step of the genotyping assay (see below). The IllumiCodes represented on the universal array consist of 1,624 artificial sequences selected to not cross-hybridize with each other or with sequences in the human genome. LSOs are synthesized with a 5' phosphate to enable ligation. Annealing reagent (OB1;

Illumina; 30 µl) and SNP-specific oligonucleotides (OPA; Illumina; 10 µl) were combined with DNA (10 µl), to a final volume of 50 µl. Annealing was carried out by ramping the temperature from 70°C to 30°C over approximately 2 hours, then holding at 30°C until the next processing step.

Oligonucleotide probe extension and ligation: Following oligonucleotide hybridization, several wash steps were performed to remove excess and mis-hybridized oligonucleotides. In a single reaction, a DNA polymerase with high specificity for a 3' match and no strand displacement or exonuclease activity was used to extend the ASO(s) that perfectly matches the target sequence at the SNP site and fill the gap between the ASO and LSO, and a DNA ligase was used to seal the nick between the extended ASO and the LSO to form PCR templates that can be amplified with universal PCR primers. Master mix for extension and ligation (MEL; Illumina; 37 µl) was added to the beads. Extension and ligation was carried out at 45°C for 15 minutes. Locus specificity is achieved by the requirement that both the ASO and LSO oligonucleotides hybridize to the same target site; extension of the appropriate ASO and ligation of the extended product to the adjacent LSO joins information about the genotype present at the SNP site to the address sequence on LSO.

PCR amplification: After extension and ligation, the beads were washed with universal buffer 1 (UB1; Illumina) to remove excess and mis-hybridized oligonucleotides. The beads were then re-suspended in 35 µl elution buffer (IP1; Illumina) and heated at 95°C for one minute to release the ligated products. The supernatant was then used in a 60 µl PCR reaction. PCR reactions were thermo-cycled as follows: 10 minutes at 37°C; 34 cycles of (35 seconds at 95°C, 35 seconds at 56°C, 2

minutes at 72°C); 10 minutes at 72°C; and cooled to 4°C for 5 minutes. The three universal PCR primers (P1, P2 and P3) were 5'-labeled with Cy3, Cy5, and biotin, respectively.

PCR product preparation: Double stranded PCR products were immobilized onto paramagnetic particles by adding 20 µl of Paramagnetic Particle B Reagent (MPB; Illumina) to each 60 µl PCR reaction, then incubated at room temperature for a minimum of 60 minutes. The bound PCR products were washed with universal buffer 2 (UB2; Illumina), and denatured by adding 30 µl 0.1 N NaOH. After 1 minute at room temperature, the released ssDNAs are neutralized with 30 µl of hybridization reagent (MH1; Illumina) and hybridized to the universal array.

Array hybridization, wash and imaging: The single-stranded, dye-labeled DNA products were hybridized to their complement bead type through their unique IllumiCode sequences. The Sentrix Array Matrix (SAM) was then washed and imaged. Specifically, arrays were hydrated in UB2 for 3 minutes at room temperature, then preconditioned in 0.1 N NaOH for 30 seconds. Arrays were returned to the UB2 reagent for at least 1 minute to neutralize the NaOH. The pre-treated arrays were exposed to the labeled ssDNA samples described above. Hybridization was conducted under a temperature gradient program from 60°C to 45°C over approximately 12 hours. The hybridization was held at 45°C until the array was processed. After hybridization, the arrays were first rinsed twice in UB2 and once with IS1 (IS1; Illumina) at room temperature with mild agitation, dried for 20 minutes, then imaged at a resolution of 0.8 microns using a BeadArray Reader (Illumina). Photomultiplier tube (PMT) settings were

optimized for dynamic range, channel balance and signal-to-noise ratio. Cy3 and Cy5 dyes were excited by lasers emitting at 550 nm and 630 nm, respectively.

Automatic genotype scoring: Based on the intensities detected from the two channels for the two respective alleles of each SNP, genotypes were called automatically using Illumina's proprietary genotyping software (GenCall). GenCall software considers multiple factors; including the distribution of beads of the same type. Outliers are rejected to ensure genotyping accuracy.

A "quality" score, the GenCall score, is calculated for each genotype call, reflecting the degree of separation between homozygote and heterozygote clusters for that SNP and the placement of the individual call within a cluster. It ranges from 0 to 1, and has been shown to correlate with the overall accuracy of the genotyping call(Fan et al., *supra*).

Exhibit 3: Multiplex Detection without Immobilization of Ligation Complexes

Annealing of assay oligonucleotides to genomic DNA: Genomic DNA (1.5 µl at 330 ng/µl) was mixed with Allele-Specific Oligonucleotides (ASOs), Locus-Specific Oligonucleotide (LSO) and master mix for extension and ligation reagent (MEL) in a reaction buffer to a final volume of 30 µl.

	ul
gDNA(300ng/ul)	1.5
5M NaCl	0.2
10x ligation buffer	3
25nM OLA mix	2.5
MEL	22.8

Annealing was carried out by ramping the temperature from 70°C to 45°C for approximately 1.5 hours.

Oligonucleotides probe extension and ligation: Since the extension and ligation enzymes were included in the annealing buffer, the extension and ligation reactions occurred simultaneously in the 70-45°C annealing step. After it reached 45°C, the tube was incubated at that temperature for an additional 15 minutes to complete the whole extension and ligation step. A DNA polymerase with high specificity for a 3' match and no strand displacement or exonuclease activity was used to extend the ASO(s) that perfectly match the target sequence at the SNP site and fill the gap between the ASO and LSO, and a DNA ligase was used to seal the nick between the extended ASO and the LSO to form PCR.

PCR amplification: After extension and ligation, a small portion of the reaction was used for PCR amplification in a 60 µl reaction.

	ul
extended and ligated products	5
H ₂ O	25
MMP(PCR reagent)	30

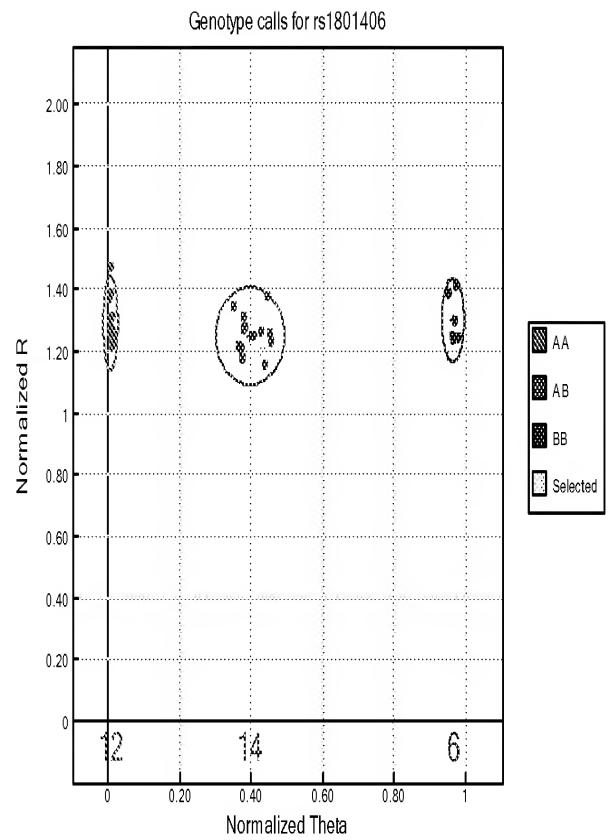
PCR reactions were thermo-cycled as follows: 10 minutes at 37°C; 34 cycles of (35 seconds at 95°C, 35 seconds at 56°C, 2 minutes at 72°C); 10 minutes at 72°C; and cooled to 4°C for 5 minutes. The three universal PCR primers (P1, P2 and P3) were 5'-labeled with Cy3, Cy5, and biotin, respectively.

PCR product preparation, array hybridization, wash, imaging, and automatic genotype scoring: Performed following the protocol described in [Exhibit 2](#).

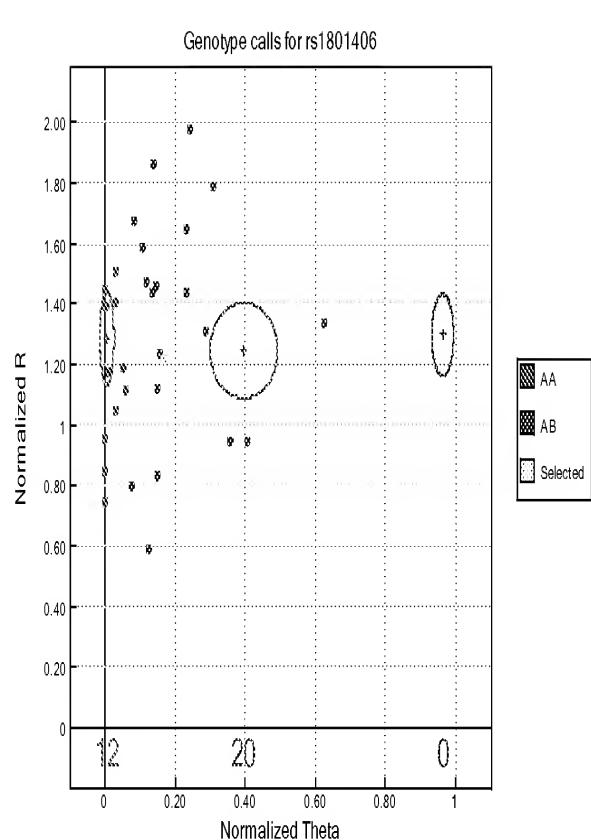
Exhibit 4

	Without Immobilization		With Immobilization	
	call rate	concordence	call rate	concordence
gDNA1	0.484	0.857	0.986	0.971
gDNA2	0.440	0.841	0.991	0.973
gDNA3	0.691	0.623	0.990	0.977
gDNA4	0.426	0.859	0.991	0.996
gDNA5	0.614	0.754	0.989	0.983
gDNA6	0.746	0.698	0.985	0.971
gDNA7	0.595	0.856	0.991	0.990
gDNA8	0.410	0.753	0.990	0.984
gDNA9	0.647	0.796	0.988	0.988
gDNA10	0.456	0.869	0.991	0.991
gDNA11	0.615	0.818	0.987	0.996
gDNA12	0.339	0.868	0.987	0.984
gDNA13	0.564	0.551	0.983	0.964
gDNA14	0.454	0.876	0.986	0.987
gDNA15	0.605	0.808	0.990	0.978
gDNA16	0.690	0.708	0.991	0.982
avg	0.548	0.783	0.989	0.982

Exhibit 5



With Immobilization



Without Immobilization

Exhibit 6

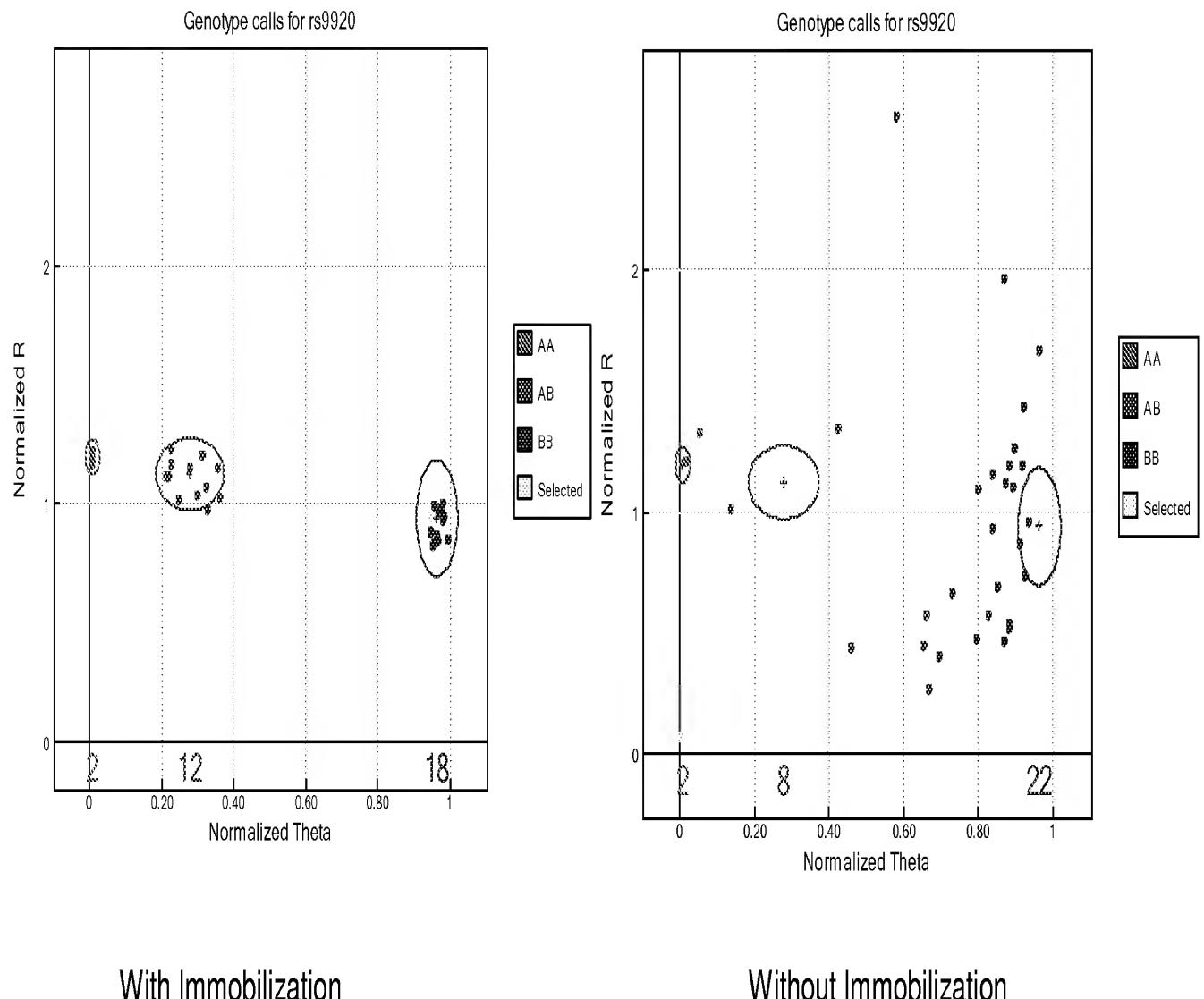


Exhibit 7

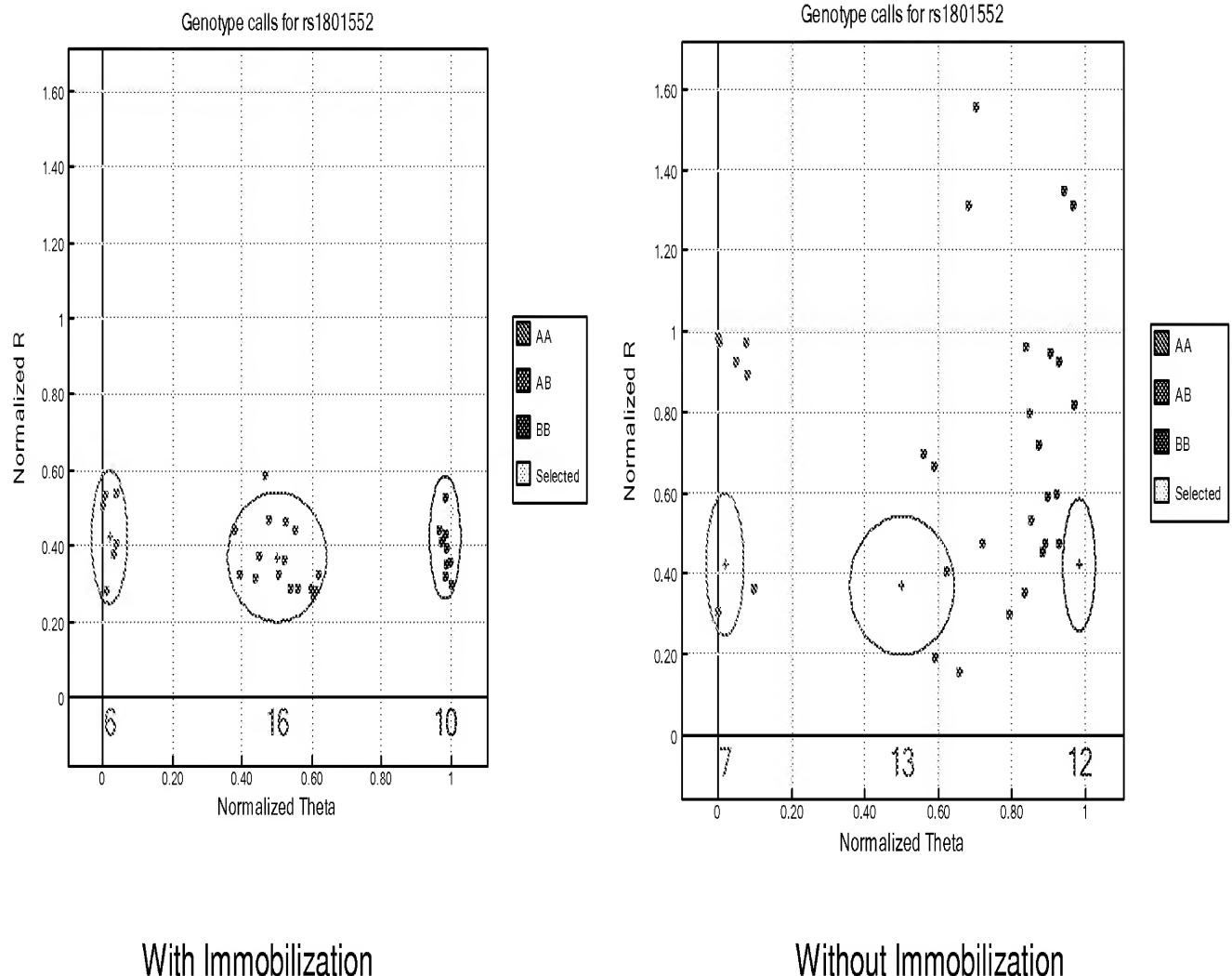
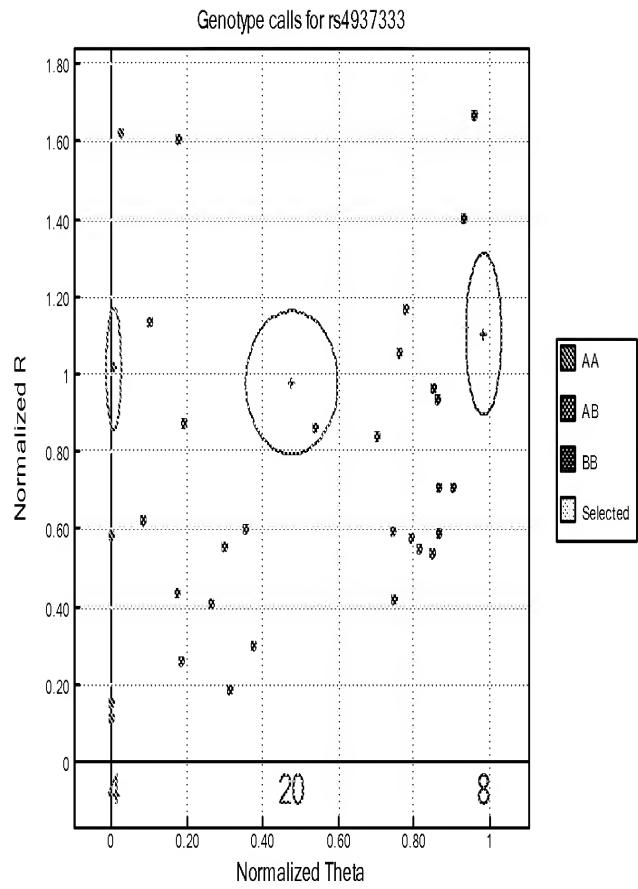
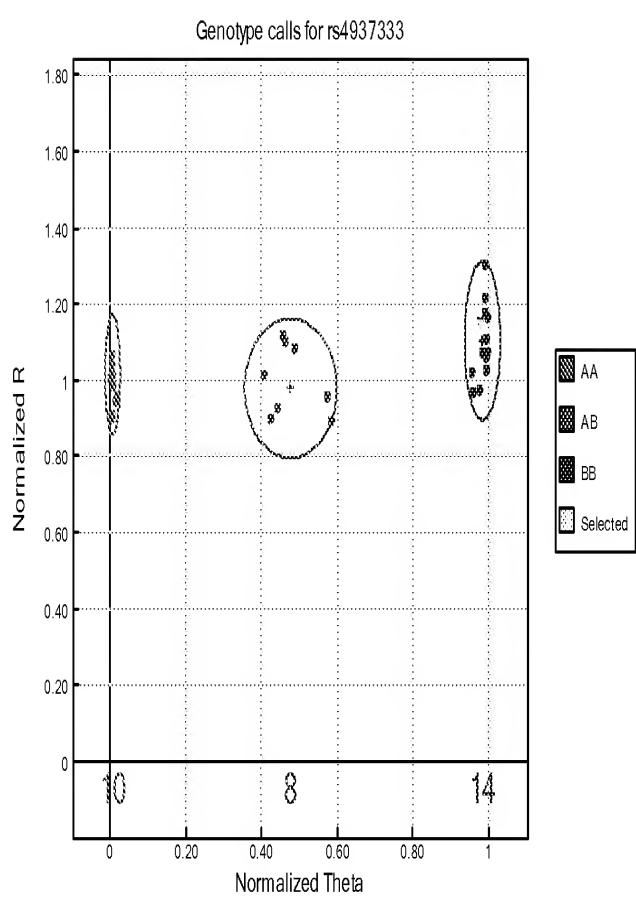


Exhibit 8



With Immobilization

Without Immobilization

Exhibit 9

